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The murine Cl/HCO₃(-) exchanger Ae3 (Slc4a3) is not required for acid-base balance but is involved in magnesium handling by the kidney

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Abstract: Background: The Slc4 family of bicarbonate transporters consists of several members, many of which are highly expressed in the kidney and play an important role in acid-base homeostasis. Among them are Ae1 (Slc4a1) and Ae2 (Slc4a2). Another member, Ae3 (Slc4a3), is suggested to be expressed in the kidney, however, its localization and impact on renal function is still unknown. Ae3 has also been implicated in the central control of breathing. Aims: Here, we analyzed the expression of Ae3 transcripts in isolated nephron segments and investigated systemic and renal acid-base homeostasis and renal electrolyte handling in the absence of Ae3, using a knock out mouse model. Methods: qPCR was used to localize Ae3 transcripts in the murine nephron, metabolic studies and whole body plethysmography to assess the role of Ae3 in renal functions. Results: Two Ae3 transcripts, the brain variant bAe3 and the cardiac variant cAe3, are expressed at low levels in the murine kidney. Although differentially distributed, they localize mostly to the distal nephron and renal collecting duct system. At baseline and after an acid challenge, mice deficient for Ae3 did not show major disturbances in renal acid-base excretion. Respiratory responses in whole body plethysmography to acid loading and CO₂ and O₂ challenges were normal. No major differences in renal electrolyte handling were discovered except for small changes in magnesium, potassium and sodium excretion after 7 days of acid loading. We therefore challenged mice with diets with high and low magnesium diets and found no differences in renal magnesium excretion but elevated expression of the Trpm6 magnesium channel in Ae3 KO mice. In conclusion, Ae3 is expressed in murine kidney at very low levels. Conclusions: Ae3 plays no role in systemic acid-base homeostasis but may modify renal magnesium handling inducing a higher expression of Trpm6.

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**The murine Cl⁻/HCO₃⁻ exchanger Ae3 (Slc4a3) is not
required for acid-base balance but is involved in
magnesium handling by the kidney**

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Keywords

Kidney • Acid-base physiology • Anion exchanger • Slc4a3 • Mouse model

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1 **ABSTRACT**

2

3 The Slc4 family of bicarbonate transporters consists of several members, many of
4 which are highly expressed in the kidney and play an important role in acid-base
5 homeostasis. Among them are Ae1 (Slc4a1) and Ae2 (Slc4a2). Another member Ae3
6 (Slc4a3) is suggested to be expressed in the kidney, however its localization and
7 impact on renal function is still unknown. Ae3 has also been implicated in the central
8 control of breathing. Here, we analyzed the expression of Ae3 transcripts in isolated
9 nephron segments and investigated systemic and renal acid-base homeostasis and
10 renal electrolyte handling in the absence of Ae3, using a knock out mouse model.
11 Two Ae3 transcripts, the brain variant *bAe3* and the cardiac variant *cAe3*, are
12 expressed at low levels in the murine kidney. Although differentially distributed, they
13 localize mostly to the distal nephron and renal collecting duct system. At baseline and
14 after an acid challenge, mice deficient for Ae3 did not show major disturbances in
15 renal acid-base excretion. Respiratory responses in whole body plethysmography to
16 acid loading and CO₂ and O₂ challenges were normal. No major differences in renal
17 electrolyte handling were discovered except for small changes in magnesium,
18 potassium and sodium excretion after 7 days of acid loading. We therefore
19 challenged mice with diets with high and low magnesium diets and found no
20 differences in renal magnesium excretion but elevated expression of the Trpm6
21 magnesium channel in Ae3 KO mice. In conclusion, Ae3 is expressed in murine
22 kidney at very low levels. Ae3 plays no role in systemic acid-base homeostasis but
23 may modify renal magnesium handling inducing a higher expression of Trpm6.

1 INTRODUCTION

2
3 The mammalian kidney serves different functions such as the regulation of
4 blood pressure, the excretion of waste products, and the maintenance of water,
5 electrolyte, mineral and acid-base homeostasis within a very narrow range. The renal
6 reabsorption and secretion of acid and base equivalents is achieved by the
7 sequential expression of different transporters and channels along the nephron [1-3].
8 Among them are several members of the Slc4 family of bicarbonate transporters and
9 the Slc26 family of anion transporters and channels [4-5]. At present, ten members of
10 the Slc4 gene family have been described and clustered into three major clades of
11 which Ae1, Ae2 and Ae3 belong to the electroneutral and sodium independent Cl^-
12 $/\text{HCO}_3^-$ exchangers [6-7]. While Ae2 is widely expressed in epithelial and non-
13 epithelial cells, Ae1 is mostly restricted to red blood cells, cardiomyocytes, renal
14 podocytes and type A intercalated cells of the renal collecting ducts (CD) [8-10]. In
15 contrast, Ae3 is predominantly found in excitable tissues, such as the central nervous
16 system, retina and heart, where it contributes to the control of intracellular pH and
17 intracellular $[\text{Cl}^-]$ [11-12]. Several Ae3 mRNA variants have been proposed, but up to
18 date, only two tissue specific polypeptide variants of Ae3 were identified, the full
19 length brain Ae3 (bAe3) isoform and the shorter N-terminal variant cardiac Ae3
20 (cAe3) [13-14].

21 In humans, AE3 mutations are not directly linked to diseases as it is the case
22 for AE1. However, the human AE3 A867D polymorphism was found to be associated
23 with the development of idiopathic generalized epilepsy (IGE) [15] and displays a
24 reduced transport activity when transiently expressed in HEK 293 cells [16]. Similarly,
25 a reduced seizure threshold upon epileptogenetic drug application was described in
26 one Ae3 deficient mouse model [17]. Furthermore, a reduced respiratory rate in
27 awake and anesthetized animals was found in the same mouse model [18]. However,
28 another mouse model with homozygous disruption of Ae3, displays functional and
29 morphological defects of the inner retina, with late onset photoreceptor death
30 resulting in blindness at the age of 4-6 months [19].

31 At present, only little is known about Ae3 expression and function in other
32 epithelial tissues, such as the gut or kidney [4, 20]. Renal Ae3 mRNA expression has

1 been described in several studies [4, 20-21]. In one study *Ae3* mRNA expression was
2 reported in isolated medullary thick ascending limbs of mouse kidney [22]. Another
3 study described protein expression of the cardiac *Ae3* isoform in arterial smooth
4 muscle of rat and mouse kidney, but not in renal tubular structures [23]. However,
5 more detailed data on the localization and function of *Ae3* in the kidney are still
6 missing.

7 Here we address the question of the epithelial *Ae3* localization and function in
8 murine kidney. We performed semi-quantitative RT-PCR on hand-dissected isolated
9 renal tubules. Furthermore, we analyzed renal function with emphasis on acid-base
10 and electrolyte handling in an *Ae3* deficient mouse model. Our data show, that renal
11 function under physiologic conditions was not affected in the absence of *Ae3*, despite
12 a differential expression of both *Ae3* transcripts along the nephron and renal
13 collecting ducts. However, *Ae3* deficient mice showed changes in magnesium
14 homeostasis and changes in the expression of renal key molecules involved in
15 magnesium transport suggesting a role of *Ae3* in magnesium handling by the kidney.

MATERIALS AND METHODS

Animals

Ae3 (Slc4a3) targeted knockout mice have been previously described [17]. Animal studies were approved by the local veterinary authority (Veterinäramt Kanton Zürich) and performed in accordance with the Swiss animal welfare law. 10-12 weeks old Ae3 WT ($Ae3^{+/+}$) and KO ($Ae3^{-/-}$) male littermates bred from heterozygote matings were placed into metabolic cages (Tecniplast, Italy) with powdered standard rodent chow GLP 3433 (Kliba Nafag, Switzerland) for basal conditions and tap water *ad libitum*. The animals were allowed to adapt to the new environment for 3 days before assessment of metabolic parameters. 24 hour urine was collected under mineral oil (Sigma, Buchs, Switzerland). 24 hour oral acid-load was carried out by addition of 150 ml of 0.33M HCl to 100 g of powdered standard rodent chow with tap water *ad libitum* [24]. Mice that received dietary magnesium challenges were primarily adapted for 7 days to a standard powder diet from SAFE (Scientific Animal Food and Engineering, France) containing standard (0.1%) Mg^{2+} in normal cages. Afterwards, the animals were fed a high (0.6%) Mg^{2+} diet for a total of 7 days and then switched to a low (0.01%) Mg^{2+} diet for 7 days. The Ca^{2+} content in all types of Mg^{2+} diets was kept constant at 0.76%. Also, all other components were kept constant in the various diets. In order to assess metabolic parameters and 24h urine samples, the mice were alternately placed in normal or metabolic cages due to the restriction by the Swiss Animal welfare law on the maximum duration of metabolic cage experiments (supplementary table S1). In a separate set of experiments, adult C57Bl/6 wildtype male mice were fed powdered standard rodent chow supplemented with $NaHCO_3$ (0.28M) for 7 days in normal cages to induce chronic metabolic alkalosis. Since the alkali loaded diet goes along with an increased sodium uptake, two groups of control animals were included in this experiment. One control group was fed a powdered standard rodent chow alone and a second group received powdered rodent chow supplemented with equimolar amounts of NaCl (0.28M) for 7 days. During the alkali loading experiments, animals were housed in normal cages.

At the end of dietary or metabolic cage experiments, mice were anesthetized with 1.5% Isoflurane/pressurized air and 500 μ l venous blood was taken with a heparinised syringe from the *V. cava caudalis* for subsequent blood gas analysis (ABL505 or ABL800, Radiometer, Copenhagen). The remaining blood was

centrifuged at 5000 rpm (4°C) for 5 minutes and the plasma was rapidly frozen until further analysis. Animals were perfused with PBS through the left ventricle to remove residual blood and kidneys were snap frozen and stored at -80°C until further use.

Plasma and urine analysis

Urinary creatinine was measured according to Jaffe's method [25]. Urinary ammonium was measured following Berthelot's protocol [26]. Plasma and urinary urea levels were measured using the diacetyl monoxime method [27] and inorganic phosphate was determined using the acid ammonium molybdate method [28]. Urinary Na⁺ and K⁺ were quantified by ionchromatography, whereas plasma and urinary Mg²⁺ and Ca²⁺ were determined with commercially available magnesium and calcium assay kits (Quantichrom DIMG-250, DICA-500), respectively. Urinary electrolytes were normalized to creatinine and presented as mean ± SEM.

RNA analysis

mRNA extraction of organs and hand-dissected isolated nephron segments with subsequent quantitative real time RT-PCR was performed as described previously [29-30]. Purity of the hand-dissected nephron segment preparation was ensured, by testing each sample for the most dominantly expressed segment specific mRNA transcripts (supplementary figure S2). Sequences of primers and probes used are listed in supplementary material (S3).

Plethysmography

Respiratory function was assessed in awake and unrestrained mice using a whole body plethysmograph (Buxco, United Kingdom). In total, 5 WT and 5 Ae3 KO male littermates were investigated. Animals were placed in separate chambers and allowed to adapt to their new environment for 30 minutes. Respiratory parameters of each condition were followed over a period of 10 minutes with averaged recordings for every minute. Baseline and recovery periods were measured at normal atmosphere. Hypercapnia was induced with controlled inflation of 5% CO₂ to the

1 chambers. Hypoxia was induced by lowering the oxygen concentration to 12% and
2 8% in the chamber air supply. In these experiments oxygen was replaced by
3 nitrogen. Respiratory function under hypercapnia was measured under standard
4 dietary conditions and after an acute (24h) acid load with HCl supplemented food. In
5 between the experiments, animals were allowed to recover for one week. Data are
6 represented as mean \pm SEM.

7 8 9 *Statistical analysis*

10 Statistical analysis was performed with GraphPad Prism software and
11 Student's t-test or ANOVA were performed where appropriate. Results with $p \leq 0.05$
12 were considered statistically significant.

RESULTS

We confirmed mRNA expression of both *Ae3* isoforms in total mouse kidney consistent with earlier reports [20-21, 31] (Fig. 1). Moreover, *bAe3* and *cAe3* are differentially expressed throughout the renal tubular segments tested. While *bAe3* expression is mostly found in the loop of Henle until the inner medullary collecting duct (IMCD), *cAe3* expression peaks in the medullary thick ascending limb (mTAL) and the outer and inner medullary collecting ducts (OMCD, IMCD). Transcripts of both isoforms were almost undetectable in the glomerulum and proximal tubule segments. In order to confirm these findings using a different method we also tried chromogenic *in-situ* hybridization, but failed to detect a specific signal in wildtype kidneys using *Ae3* KO kidneys as control for specificity (data not shown), which might be due to the relatively low mRNA expression levels as indicated by the qPCR experiments. However, the localization of control genes with known renal distribution, including *Slc5a2* (*Sgt2*) and *Slc12a1* (*Nkcc2*) yielded the expected localization confirming that the procedure worked (data not shown). We were unable to confirm the localization of *bAe3* or *cAe3* on protein level, since several antibodies raised against *Ae3* did not give specific signals in mouse kidneys used for indirect immunofluorescence, immunohistochemistry or immunoblotting (data not shown). However, specific signals were obtained with several of these antibodies in brain and heart from WT and *Ae3* KO mice (data not shown) most likely reflecting a very low abundance of *Ae3* protein in murine kidney.

Next, we examined the impact of the absence of *Ae3* on renal acid-base and electrolyte handling by using an *Ae3* deficient mouse model. First, we compared baseline blood and urine parameters of *Ae3*^{+/+} and *Ae3*^{-/-} male littermates at the age of 12 weeks adapted to metabolic cages and fed a standard rodent chow with access to water *ad libitum* (Table 1). Despite a mildly but significantly lower blood lactate level in *Ae3* deficient mice, other differences between the genotypes were not detected. No respiratory alterations were observed as assessed by venous blood gas analysis and whole body plethysmography (Fig. 2 A-D). Venous blood gas analysis performed at baseline without any dietary or respiratory challenges showed similar blood pH, pCO₂ and pO₂ in both genotypes (Table 1). Respiratory analysis at baseline demonstrated similar respiratory frequency, and tidal volume (Fig. 2 A-D). When *Ae3*^{+/+} and *Ae3*^{-/-} were challenged with 5% CO₂, both genotypes responded

1 with a transient increase in respiratory frequency and a profound increase in tidal
2 volume, but no difference was observed between both groups. Similarly, when both
3 groups of mice were exposed to reduced oxygen content (12% and 8%), tidal volume
4 was increased to a similar extent. In a last set of experiment, mice were given 1 day
5 of HCl in the food to induce metabolic acidosis and then challenged with inspiratory
6 hypercapnia (5% CO₂), but both groups of mice showed a similar response. Thus,
7 absence of Ae3 in this mouse model does not alter the respiratory response to
8 changes in ambient O₂ or CO₂, as well as to short term metabolic acidosis.
9 Therefore, we continued with renal phenotyping of Ae3 KO mice with dietary
10 challenges targeting acid-base homeostasis.

11 Wildtype and Ae3 KO mice received oral acid challenges for 1 to 7 days. Mice
12 with a deficiency for the Cl⁻/HCO₃⁻ exchanger Ae3 did not show any significant
13 differences in blood and urinary pH, pCO₂ or HCO₃⁻ compared to their wildtype
14 littermates, when subjected to an acute or chronic dietary acid load for 24 hours or 7
15 days, respectively (Table 2 and 3). There were no differences in blood and urinary
16 electrolyte concentrations of Ae3 WT and KO mice after acute dietary acid
17 challenges except for plasma sodium after 7 days of acid challenge (Table 2). As
18 expected, a transient reduction of blood Pi levels following acute dietary acid loads
19 was observed for both genotypes (Tables 2 and 3). Urinary phosphate excretion
20 increased in both genotypes with the acid challenge as expected. Metabolic acidosis
21 in mice inhibits the function of the renal NaPi-IIa cotransporter and thereby increases
22 urinary excretion of phosphate [32]. However, a significant increase in the anion gap
23 in blood of Ae3 KO mice with acute acid load was detected, which was even more
24 pronounced after a chronic 7 days acid load (Table 3). Moreover, a chronic dietary
25 acid load revealed additional significant differences in blood and urine of Ae3 KO
26 mice compared to Ae3 WT littermates (Table 3). As previously shown, induction of
27 metabolic acidosis leads to an increase in urinary aldosterone and Na⁺ excretion in
28 mice and rats [29]. Ae3 KO mice didnot increase urinary Na⁺ excretion and showed
29 mildly elevated Na⁺ concentrations in the blood, as compared to Ae3 WT.
30 Additionally, urinary K⁺ and Mg²⁺ levels were significantly reduced in Ae3 KO mice
31 exposed to chronic dietary acid loads. In order to identify transport proteins that are
32 involved in renal handling of calcium and magnesium and that may be modulated by
33 the absence of Ae3, we performed qPCR. Assessment of renal *Ae1* Cl⁻/HCO₃⁻

1 exchanger and the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter *Nkcc2* mRNA expression [32[33] did not
2 show any altered expression (Table 6).

3 The reduced urinary Mg^{2+} excretion in the presence of a chronic dietary acid
4 load of Ae3 KO mice (Table 3) and the expression of Ae3 mRNA in nephron
5 segments involved in magnesium handling (Fig. 1 C) suggested a link of Ae3 to
6 magnesium homeostasis. Hence, mice were challenged with high (0.6%) and low
7 (0.01%) Mg^{2+} diets for 7 days. As listed in Tables 4 and 5, the adaptation of
8 electrolyte and acid-base parameters in blood and urine of Ae3 WT and Ae3 KO
9 littermates to chronic dietary Mg^{2+} challenges was similar. However, renal mRNA
10 expression of the apical Mg^{2+} and Ca^{2+} channels *Trpm6* and *Trpv5* [34-35] was
11 significantly increased in Ae3 deficient mice compared to WT after 7 days of low Mg^{2+}
12 diet, while claudin 16 (*Cldn16*) [36-37] mRNA was significantly down regulated (Table
13 6). Neither claudin 19 (*Cldn19*), nor basolaterally expressed cyclin M2 (*Cnnm2*),
14 involved in renal Mg^{2+} handling, were altered [36-38] (Table 6).

15 Finally, transcript levels of *bAe3* and *cAe3* were assessed in *Ae3*^{+/+} kidneys
16 following standard diet, 24 hour acute acid load, 7 days low Mg^{2+} diet and 7 days
17 chronic alkali load (Fig. 3). Both *Ae3* isoforms were downregulated upon dietary
18 induced metabolic acidosis (Fig. 3 A, B). However, a regulation of *Ae3* mRNA was
19 not observed in kidneys of C57Bl/6 mice after a chronic dietary alkali load (Fig. 3 C,
20 D). Also, low Mg^{2+} diet for 7 days downregulated *bAe3* but not *cAe3* mRNA (Fig. 3
21 A).

DISCUSSION

Expression of *bAe3* and *cAe3* transcripts in mouse kidney has been previously shown [4, 20-21]. However, localization to a specific nephron or collecting duct segment was still missing. We confirm expression of both Ae3 variants in mouse kidney and demonstrate that both isoforms are differentially distributed within the kidney, with *bAe3* being the dominant isoform expressed in total kidney. While *bAe3* is found at similar levels from the loop of Henle until the IMCD, *cAe3* seems to be restricted to the mTAL, OMCD and IMCD. However, presence of the respective proteins could not be confirmed, due to unavailability of specific antibodies and possibly low abundance of the protein.

Based on the chloride/bicarbonate exchanger function of Ae3 one may expect a contribution of Ae3 to renal acid-base and electrolyte handling [6-7]. Testing this hypothesis the acid-base status of Ae3 WT and KO mice was analyzed under standard conditions and after short and longer acid challenges. Apart from a modest but significantly reduced blood lactate concentration, acid-base and electrolyte values in blood and urine of Ae3 deficient mice appeared normal (Table 1). Since bodyweight, blood pH, pCO₂ and pO₂ were not altered, the origin of the mildly reduced blood lactate is not clear. Small changes in energy metabolism or tissue oxygenation cannot be ruled out. However, blood lactate levels were not affected by acute dietary acid loads (Table 2). Additionally, the breathing pattern of 12 week old, male, conscious and unrestrained Ae3^{+/+} and Ae3^{-/-} littermates was analyzed by whole body plethysmography during standard conditions (standard rodent chow, normoxia), hypoxia and hypercapnia, as well as after an acute dietary acid load in the setting of normoxia and hypercapnia. In contrast to previous findings [18], we could not observe significant differences in the breathing pattern and breathing parameters of Ae3 deficient mice compared to Ae3 WT littermates in any condition (Fig. 2). However, our observation of normal respiratory responses to hypercapnia and hypoxia is in line with the finding of normal blood gases throughout all metabolic cage experiments that were performed in this study. Of note, the previous study reporting differences between wildtype and Ae3 knockout mice did not use littermates and used mice of different age and sex, which may have introduced a bias into the experiments [18].

1 Challenging Ae3 deficient mice with acute or chronic dietary acid load did also not
2 reveal major alterations in renal acid-base or electrolyte handling (Tables 2 and 3).
3 Again, blood and urinary pH, $p\text{CO}_2$ and HCO_3^- were not significantly different between
4 the genotypes. Surprisingly, blood anion gap was significantly increased in Ae3 KO
5 mice subjected to acute and chronic dietary acid loads. While the ions determining
6 the anion gap were not significantly altered after an acute dietary acid load in Ae3 KO
7 mice compared to Ae3 WT, blood and urinary Na^+ , as well urinary K^+ and Mg^{2+}
8 became significantly altered in Ae3 KO animals with a chronic induced metabolic
9 acidosis (Table 3). However, the difference in anion gap is small and apparently did
10 not affect acid-base homeostasis as judged from venous blood pH, $p\text{CO}_2$ and
11 bicarbonate levels.

12 Mice lacking Ae3 showed dysregulation of the renal Ca^{2+} - and Mg^{2+} -channels Trpv5
13 and Trpm6 as well as of Cldn16 which was most obvious during the low Mg^{2+} diet.
14 Cldn16 is required for renal calcium and magnesium reabsorption as evident from
15 patients with rare mutations in claudin 16 [39-41]. Thus, in theory, loss of Ae3 in the
16 TAL may affect Cldn16 expression and function and stimulate the expression of
17 Trpv5 and Trpm6 expression and function in the late distal convoluted tubule and
18 connecting tubule. Whether claudins in kidney are regulated by local or systemic pH
19 changes has not been systematically investigated. Similarly, Ae3 mediated $\text{Cl}^-/\text{HCO}_3^-$
20 exchange and Nkcc1 (Slc12a1) mediated $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport in cardiomyocytes
21 affect Ca^{2+} transport by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger Ncx and phospholamban (Pln), as
22 well as the performance of the regulator phosphatases PP1 and PP2A [42]. Notably,
23 another member of the Slc12 family, Nkcc2 (Slc12a2) and Ncx are also expressed in
24 the kidney and involved in renal Na^+ and Ca^{2+} handling [43]. While Nkcc2 is
25 expressed in the thick ascending limb (TAL), Ncx localizes to the DCT and CNT [43].
26 As illustrated in Fig. 1, both Ae3 transcripts are present in the loop of Henle, the site
27 of Nkcc2 expression, with cAe3 being predominantly in the mTAL. Furthermore, bAe3
28 mRNA is detected in the DCT and CCD (including CNT) meeting the localization of
29 renal Ncx. Furthermore, renal Na^+ and Mg^{2+} handling is altered in Ae3 deficient mice
30 subjected to chronic dietary acid loads (Table 3). However, renal Ncx and Nkcc2
31 mRNA expression was not altered in dietary challenged Ae3 deficient mice compared
32 to WT (Table 6). Unfortunately, protein abundance of Cldn16, Trpm6 and Trpv5 could
33 not be determined due to lack of specific antibodies suitable for immunoblotting (data
34 not shown). However, adaptation of acid-base and electrolyte concentrations in blood

1 and urine to either high Mg^{2+} diet (Table 4) or low Mg^{2+} diet (Table 5) was similar in
2 Ae3 WT and Ae3 KO animals. Finally, a comparison of Ae3 mRNA expression in Ae3
3 WT mice shows only a mild reduction of *bAe3* and *cAe3* transcripts upon dietary
4 Mg^{2+} restriction, while both isoforms are strongly regulated in response to acute
5 dietary acid loads (Fig. 3).

6
7 In summary, our data show that the both variants of Ae3, *bAe3* and *cAe3*, are
8 expressed at low levels in murine kidney and localize mostly to segments involved in
9 the fine tuning of electrolyte, acid-base and mineral excretion. No obvious alteration
10 in the renal excretion of acid and the control of systemic acid-base balance was
11 found in mice lacking Ae3. Moreover, handling of electrolytes showed also no major
12 disturbances and only a mild difference in the renal excretion of magnesium under
13 acid-loading prompted us to test high and low magnesium diets. Also under these
14 diets, Ae3 KO mice displayed no difference in plasma and urine magnesium levels
15 but may require different levels of expression of *Cldn16*, *Trpv5* and *Trpm6* to
16 maintain magnesium homeostasis during low magnesium challenges. Whether the
17 upregulation of *Trpv5* and *Trpm6* is a direct consequence of reduced *Cldn16*
18 expression and activity or directly linked to absence of Ae3 remains speculative.
19 Nevertheless, our experiments demonstrate that Ae3 plays no major role in renal
20 acid-base, electrolyte and mineral handling.

21 22 **Acknowledgements**

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24 challenged with dietary alkali loads. The use of the Zurich Integrative Rodent
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27 Wagner.

FIGURE LEGENDS

Table 1

Blood and urine parameters of $Ae3^{+/+}$ and $Ae3^{-/-}$ mice under baseline conditions

Food and water intake as well as urine and feces output were monitored in metabolic cages under baseline conditions. At the end of the observational period, venous blood and 24 hrs urine were collected and analyzed. $n = 9 Ae3^{+/+}$ and $7 Ae3^{-/-}$ mice, $*p < 0.05$

Table 2

Blood and urine parameters of $Ae3^{+/+}$ and $Ae3^{-/-}$ mice with 1 day acid load

Food and water intake as well as urine and feces output were monitored in metabolic cages after 1 day of HCl added to food to induce metabolic acidosis. Venous blood and 24 hrs urine were collected and analyzed. $n = 5 Ae3^{+/+}$ and $5 Ae3^{-/-}$ mice, $*p < 0.05$

Table 3

Blood and urine parameters of $Ae3^{+/+}$ and $Ae3^{-/-}$ mice with 7 days acid load

Food and water intake as well as urine and feces output were monitored in metabolic cages after 7 days of HCl added to food to induce metabolic acidosis. Venous blood and 24 hrs urine were collected and analyzed. $n = 5 Ae3^{+/+}$ and $8 Ae3^{-/-}$ mice, $*p < 0.05$, $**p < 0.01$

Table 4

Urine parameters of *Ae3^{+/+}* and *Ae3^{-/-}* mice with 7 days high Mg^{2+} diet

Food and water intake as well as urine and feces output were monitored in metabolic cages after 7 days of high magnesium diet (0.6% Mg^{2+}) added to food. 24 hrs urine was collected and analyzed. n = 7 *Ae3^{+/+}* and 9 *Ae3^{-/-}* mice.

Table 5

Blood and urine parameters of *Ae3^{+/+}* and *Ae3^{-/-}* mice with 7 days low Mg^{2+} diet

Food and water intake as well as urine and feces output were monitored in metabolic cages after 7 days of low magnesium diet (0.01% Mg^{2+}) added to food. Venous blood and 24 hrs urine were collected and analyzed. n = 7 *Ae3^{+/+}* and 9 *Ae3^{-/-}* mice.

Table 6

Relative mRNA expression of genes involved in renal calcium and magnesium handling under different dietary conditions

Semi-quantitative RT-PCR was performed in total kidneys from *Ae3^{+/+}* and *Ae3^{-/-}* mice receiving either standard diet (n = 9 *Ae3^{+/+}* and 7 *Ae3^{-/-}* mice), acid challenges (1 day acid load, n = 5 *Ae3^{+/+}* and 5 *Ae3^{-/-}* mice; 7 days acid load, n = 5 *Ae3^{+/+}* and 8 *Ae3^{-/-}* mice), or low magnesium diet (n = 7 *Ae3^{+/+}* and 9 *Ae3^{-/-}* mice). n.d. = not determined; *p < 0.05 ; **p < 0.01

Figure 1

Total mRNA expression of *Ae3* isoforms in isolated tubules and total mouse kidney, brain and heart. Total mRNA expression of *bAe3* and *cAe3* was determined by semi-quantitative real time RT-PCR in (A, B) brain, heart and kidney (n = 3). (C) Representative experiment (from 3 qualitatively similar experiments) for the expression of *bAe3* and *cAe3* mRNA in hand-dissected isolated renal tubules and total kidney Glom, Glomeruli; S1/2, convoluted proximal tubule; S3, straight proximal tubule; thin limb, medullary thin descending limb of loop of Henle; mTAL, medullary

thick ascending limb of loop of Henle; DCT, distal convoluted tubule; CCD, cortical collecting duct including connecting tubule; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct.

Figure 2

Whole body plethysmography of wildtype and Ae3 deficient mice challenged with hypercapnia and hypoxia with normal acid-base metabolism and hypercapnia in addition to acute metabolic acidosis. Respiratory frequency (f) and tidal volume (TV) of wildtype (WT) and Ae3 knockout (KO) mice were recorded at normal acid-base metabolism and atmosphere and during hypercapnic challenges with 5% CO₂ (**A, B**). Additionally, mice with normal acid-base metabolism were challenged with stepwise hypoxia (**C, D**) by reducing the oxygen content in the record chamber to 12% and 8%. Furthermore respiratory response (**E, F**) to hypercapnia (5% CO₂) was recorded in mice challenged with an acute dietary acid load for 24 hours. f (bpm), respiratory frequency (breath per minute); TV (tidal volume)

Figure 3

Regulation of Ae3 transcripts upon dietary acid or magnesium challenge. The mRNA abundance of *bAe3* (**A, C**) and *cAe3* (**B, D**) was assessed in *Ae3*^{+/+} kidneys after acute dietary acid load for 24 hours (1d HCl) and 7 days low Mg²⁺ diet (7d low Mg²⁺) (**A, B**) or challenged with chronic metabolic alkali loads (7d NaHCO₃⁻) (**C, D**).
* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; n.s. = not significant

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Table 1. Blood and urine parameters of *Ae3^{+/+}* and *Ae3^{-/-}* mice under baseline conditions

Baseline	<i>Ae3^{+/+}</i>	<i>Ae3^{-/-}</i>
Bodyweight (g)	27.2 ± 0.6	28.0 ± 0.7
H ₂ O Intake (g/g BW)	0.19 ± 0.01	0.17 ± 0.01
Food Intake (g/g BW)	0.18 ± 0.01	0.16 ± 0.01
Stool (g/g BW)	0.04 ± 0.01	0.04 ± 0.01
Urine (g/g BW)	0.04 ± 0.01	0.04 ± 0.01
Blood		
pH	7.36 ± 0.01	7.36 ± 0.01
pCO ₂ (mmHG)	41.2 ± 1.4	41.2 ± 1.7
pO ₂ (mmHG)	46.5 ± 0.9	46.6 ± 0.9
HCO ₃ ⁻ (mM)	22. ± 0.2	22.8 ± 0.
Na ⁺ (mM)	145.2 ± 0.	145.7 ± 0.6
K ⁺ (mM)	3.8 ± 0.1	3.7 ± 0.1
Cl ⁻ (mM)	109.4 ± 0.7	111.0 ± 1.3
Lactate (mM)	2.9 ± 0.2	2.3 ± 0.2 *
Anion Gap	13.2 ± 0.6	11.9 ± 1.5
Hematocrit (%)	42.8 ± 0.3	43.1 ± 0.5
Plasma		
P _i (mM)	2.3 ± 0.1	2.2 ± 0.1
Mg ²⁺ (mM)	0.64 ± 0.01	0.64 ± 0.02
Ca ²⁺ (mM)	3.75 ± 0.1	3.47 ± 0.2
Urea (mg/dl)	28.0 ± 1.4	25.3 ± 0.6
Urine		
Creatinine (mg/dl)	62.9 ± 4.4	67.7 ± 9.1
pH	6.02 ± 0.02	5.98 ± 0.03
HCO ₃ ⁻ (mM)/Crea (mg/dl)	0.007 ± 0.001	0.005 ± 0.001
Na ⁺ (mM)/Crea (mg/dl)	2.6 ± 0.2	2.0 ± 0.2
K ⁺ (mM)/Crea (mg/dl)	9.2 ± 0.8	8.8 ± 0.9
Ca ²⁺ (mM)/Crea (mg/dl)	0.1 ± 0.1	0.1 ± 0.1
Mg ²⁺ (mM)/Crea (mg/dl)	0.4 ± 0.1	0.5 ± 0.1
P _i (mM)/Crea (mg/dl)	1.9 ± 0.2	1.9 ± 0.1
Urea (mg/dl)/Crea (mg/dl)	108.0 ± 7.0	133.0 ± 13.5
NH ₃ /NH ₄ ⁺ (mM)/Crea (mg/dl)	0.7 ± 0.1	0.8 ± 0.1

Table 2. Blood and urine parameters of *Ae3^{+/+}* and *Ae3^{-/-}* mice with 1 day acid load

1 day acid load	<i>Ae3^{+/+}</i>	<i>Ae3^{-/-}</i>
Bodyweight (g)	30.8 ± 1.0	29.9 ± 0.6
H ₂ O Intake (g/g BW)	0.08 ± 0.02	0.16 ± 0.04
Food Intake (g/g BW)	0.15 ± 0.01	0.14 ± 0.01
Stool (g/g BW)	0.01 ± 0.01	0.01 ± 0.01
Urine (g/g BW)	0.10 ± 0.03	0.13 ± 0.03
Blood		
pH	7.15 ± 0.03	7.11 ± 0.02
pCO ₂ (mmHG)	42.6 ± 0.5	40.1 ± 1.1
pO ₂ (mmHG)	47.4 ± 3.4	52.3 ± 4.1
HCO ₃ ⁻ (mM)	14.4 ± 1.1	12.2 ± 0.9
Na ⁺ (mM)	147.0 ± 1.6	150.4 ± 0.7
K ⁺ (mM)	3.8 ± 0.1	3.9 ± 0.1
Cl ⁻ (mM)	121.8 ± 2.8	125.6 ± 0.9
Lactate (mM)	1.7 ± 0.2	1.8 ± 0.1
Anion Gap	10.6 ± 0.3	12.6 ± 0.6 *
Hematocrit (%)	44.8 ± 0.9	42.3 ± 0.7
Plasma		
P _i (mM)	1.4 ± 0.1	1.2 ± 0.1
Mg ²⁺ (mM)	0.65 ± 0.01	0.67 ± 0.02
Ca ²⁺ (mM)	3.33 ± 0.42	3.65 ± 0.04
Urine		
Creatinine (mg/dl)	14.1 ± 1.8	10.6 ± 2.6
pH	5.64 ± 0.06	5.79 ± 0.09
HCO ₃ ⁻ (mM)/Crea (mg/dl)	0.015 ± 0.004	0.039 ± 0.013
Na ⁺ (mM)/Crea (mg/dl)	5.3 ± 0.5	4.9 ± 1.1
K ⁺ (mM)/Crea (mg/dl)	10.9 ± 0.1	10.8 ± 1.8
Ca ²⁺ (mM)/Crea (mg/dl)	0.2 ± 0.1	0.3 ± 0.1
Mg ²⁺ (mM)/Crea (mg/dl)	1.0 ± 0.1	0.9 ± 0.3
P _i (mM)/Crea (mg/dl)	3.3 ± 0.2	3.4 ± 0.7
NH ₃ /NH ₄ ⁺ (mM)/Crea (mg/dl)	10.4 ± 1.3	9.7 ± 3.1

Table 3. Blood and urine parameters of *Ae3^{+/+}* and *Ae3^{-/-}* mice with 7 days acid load

7 days acid load	<i>Ae3^{+/+}</i>	<i>Ae3^{-/-}</i>
Bodyweight (g)	23.5 ± 0.5	22.6 ± 0.4
H ₂ O Intake (g/g BW)	0.09 ± 0.01	0.21 ± 0.05
Food Intake (g/g BW)	0.44 ± 0.02	0.39 ± 0.02
Urine (g/g BW)	0.16 ± 0.03	0.19 ± 0.03
Blood		
pH	7.32 ± 0.03	7.26 ± 0.03
pCO ₂ (mmHG)	38.5 ± 0.8	38.2 ± 1.5
pO ₂ (mmHG)	66.1 ± 3.9	54.8 ± 3.4
HCO ₃ ⁻ (mM)	19.2 ± 1.4	16.3 ± 0.9
Na ⁺ (mM)	146.4 ± 2.8	151.5 ± 0.5 **
K ⁺ (mM)	4.1 ± 0.2	3.9 ± 0.1
Cl ⁻ (mM)	120.8 ± 1.2	121.1 ± 1.4
Anion Gap	6.4 ± 3.2	14.0 ± 0.6 *
Plasma		
P _i (mM)	2.6 ± 0.1	2.6 ± 0.2
Mg ²⁺ (mM)	0.72 ± 0.03	0.76 ± 0.03
Ca ²⁺ (mM)	2.73 ± 0.40	3.53 ± 0.37
Urine		
Creatinine (mg/dl)	11.5 ± 0.7	11.2 ± 1.4
pH	5.63 ± 0.06	5.57 ± 0.04
HCO ₃ ⁻ (mM)/Crea (mg/dl)	0.005 ± 0.001	0.006 ± 0.001
Na ⁺ (mM)/Crea (mg/dl)	4.7 ± 0.5	2.9 ± 0.2 **
K ⁺ (mM)/Crea (mg/dl)	17.5 ± 1.3	12.9 ± 0.7 **
Ca ²⁺ (mM)/Crea (mg/dl)	0.9 ± 0.1	0.7 ± 0.1
Mg ²⁺ (mM)/Crea (mg/dl)	1.6 ± 0.2	1.2 ± 0.1 *
P _i (mM)/Crea (mg/dl)	3.0 ± 0.4	3.0 ± 0.2
NH ₃ /NH ₄ ⁺ (mM)/Crea (mg/dl)	27.8 ± 5.8	24.2 ± 1.4

Table 4. Urine parameters of *Ae3^{+/+}* and *Ae3^{-/-}* mice with 7 days high Mg^{2+} diet

7 days high Mg^{2+} diet	<i>Ae3^{+/+}</i>	<i>Ae3^{-/-}</i>
Bodyweight (g)	28.8 ± 1.5	27.6 ± 0.5
H ₂ O Intake (g/g BW)	0.23 ± 0.02	0.25 ± 0.02
Food Intake (g/g BW)	0.12 ± 0.01	0.12 ± 0.01
Urine (g/g BW)	0.11 ± 0.02	0.13 ± 0.01
Urine		
Creatinine (mg/dl)	8.5 ± 0.8	7.6 ± 0.7
pH	6.51 ± 0.04	6.58 ± 0.07
Na ⁺ (mM)/Crea (mg/dl)	27.7 ± 1.7	28.5 ± 1.5
K ⁺ (mM)/Crea (mg/dl)	8.1 ± 0.8	7.5 ± 0.4
Ca ²⁺ (mM)/Crea (mg/dl)	0.3 ± 0.1	0.3 ± 0.1
Mg ²⁺ (mM)/Crea (mg/dl)	7.0 ± 1.2	6.3 ± 0.9

Table 5. Blood and urine parameters of *Ae3^{+/+}* and *Ae3^{-/-}* mice with 7 days low Mg^{2+} diet

7 days low Mg^{2+} diet	<i>Ae3^{+/+}</i>	<i>Ae3^{-/-}</i>
Bodyweight (g)	29.7 ± 0.5	30.6 ± 0.5
H ₂ O Intake (g/g BW)	0.15 ± 0.01	0.17 ± 0.02
Food Intake (g/g BW)	0.13 ± 0.04	0.09 ± 0.01
Urine (g/g BW)	0.10 ± 0.01	0.12 ± 0.01
Blood		
pH	7.31 ± 0.01	7.32 ± 0.01
pCO ₂ (mmHG)	40.0 ± 1.2	40.8 ± 0.8
pO ₂ (mmHG)	45.0 ± 2.4	43.9 ± 2.4
HCO ₃ ⁻ (mM)	19.5 ± 0.3	20.2 ± 0.4
Na ⁺ (mM)	146.1 ± 0.3	146.8 ± 0.3
K ⁺ (mM)	3.6 ± 0.1	3.7 ± 0.1
Cl ⁻ (mM)	113.0 ± 0.7	113.2 ± 0.5
Lactate (mM)	2.8 ± 0.2	2.6 ± 0.2
Anion Gap	13.5 ± 0.9	13.4 ± 0.4
Hematocrit (%)	44.5 ± 0.6	44.84 ± 0.72
Plasma		
Mg^{2+} (mM)	0.51 ± 0.02	0.45 ± 0.02
Ca^{2+} (mM)	3.25 ± 0.36	3.37 ± 0.22
Urine		
Creatinine (mg/dl)	8.6 ± 0.7	8.0 ± 0.7
pH	5.80 ± 0.03	5.77 ± 0.01
Na ⁺ (mM)/Crea (mg/dl)	21.5 ± 1.2	22.6 ± 1.9
K ⁺ (mM)/Crea (mg/dl)	9.2 ± 0.6	10.3 ± 1.2
Ca^{2+} (mM)/Crea (mg/dl)	0.1 ± 0.1	0.1 ± 0.1
Mg^{2+} (mM)/Crea (mg/dl)	0.1 ± 0.1	0.1 ± 0.1

Table 6. Relative mRNA expression of selected genes involved in renal calcium and magnesium handling at different dietary conditions

% mRNA abundance	Baseline		1 day acid load		7 days acid load		7 days low Mg ²⁺ diet	
	<i>Ae3</i> ^{+/+}	<i>Ae3</i> ^{-/-}	<i>Ae3</i> ^{+/+}	<i>Ae3</i> ^{-/-}	<i>Ae3</i> ^{+/+}	<i>Ae3</i> ^{-/-}	<i>Ae3</i> ^{+/+}	<i>Ae3</i> ^{-/-}
<i>Ae1</i>	100.0 ± 27.1	90.1 ± 34.7	100.0 ± 10.8	107.5 ± 7.3	n.d.	n.d.	n.d.	n.d.
<i>Cnnm2</i>	100.0 ± 7.6	106.2 ± 19.5	100.0 ± 4.0	94.7 ± 5.2	100.0 ± 4.2	78.7 ± 4.2	100.0 ± 4.2	92.4 ± 3.8
<i>Trpm6</i>	100.0 ± 22.7	77.5 ± 19.3	100.0 ± 8.8	90.0 ± 8.7	100.0 ± 9.7	71.5 ± 5.9 *	100.0 ± 12.0	178.0 ± 16.8 **
<i>Trpv5</i>	100.0 ± 17.2	94.7 ± 17.3	100.0 ± 8.8	125.8 ± 10.6	100.0 ± 5.1	116.0 ± 8.8	100.0 ± 5.7	134.5 ± 10.6 *
<i>Cldn16</i>	n.d.	n.d.	100.0 ± 3.8	115.6 ± 12.1	100.0 ± 4.2	78.2 ± 4.0 **	n.d.	n.d.
<i>Cldn19</i>	n.d.	n.d.	100.0 ± 11.9	118.4 ± 16.6	100.0 ± 9.7	82.1 ± 4.6	n.d.	n.d.
<i>Ncx</i>	n.d.	n.d.	100.0 ± 10.5	113.8 ± 20.5	100.0 ± 5.6	89.0 ± 5.1	n.d.	n.d.
<i>Nkcc2</i>	n.d.	n.d.	100.0 ± 5.9	108.4 ± 12.6	100.0 ± 6.4	89.3 ± 4.1	n.d.	n.d.

Figure 1

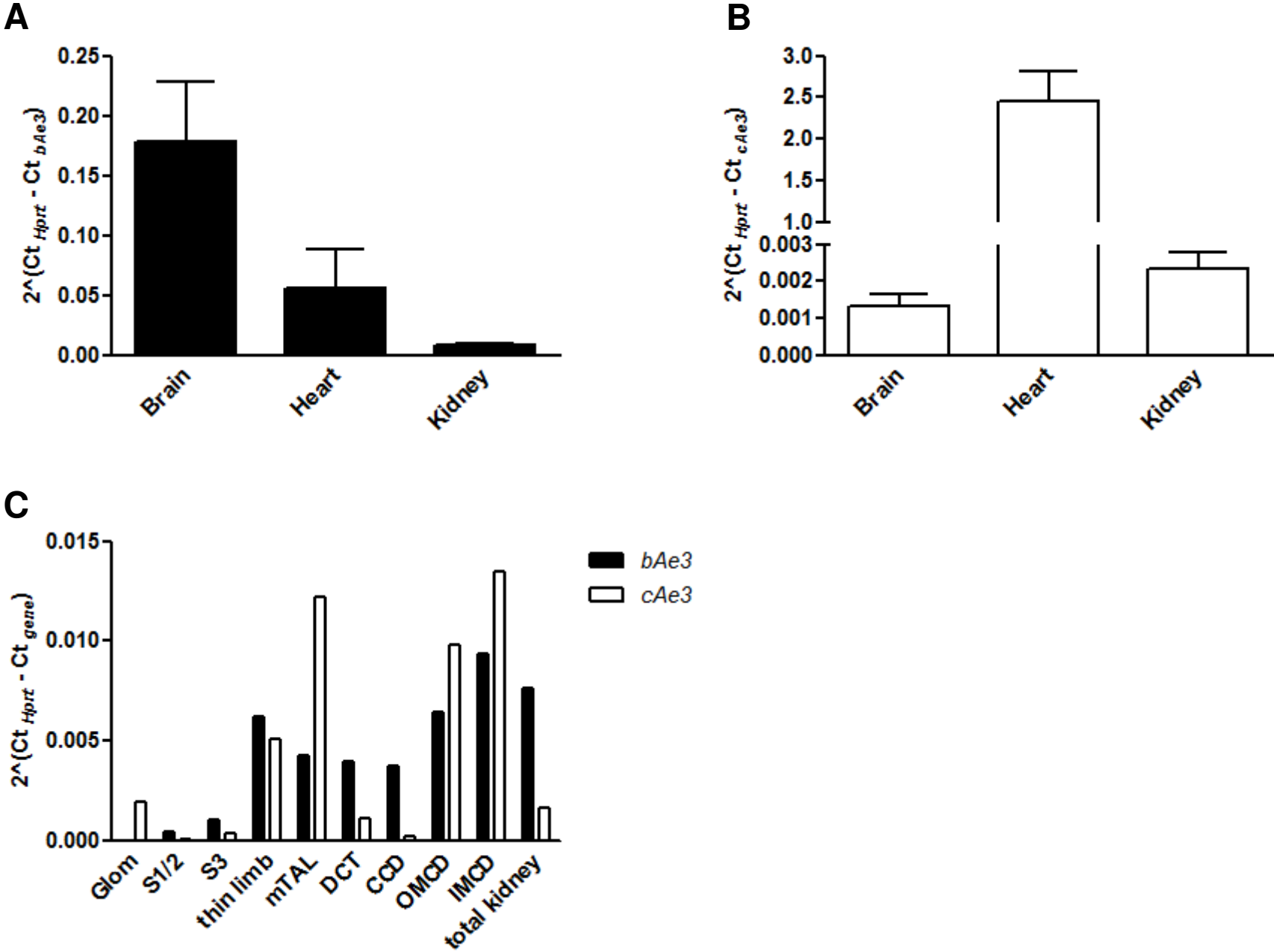
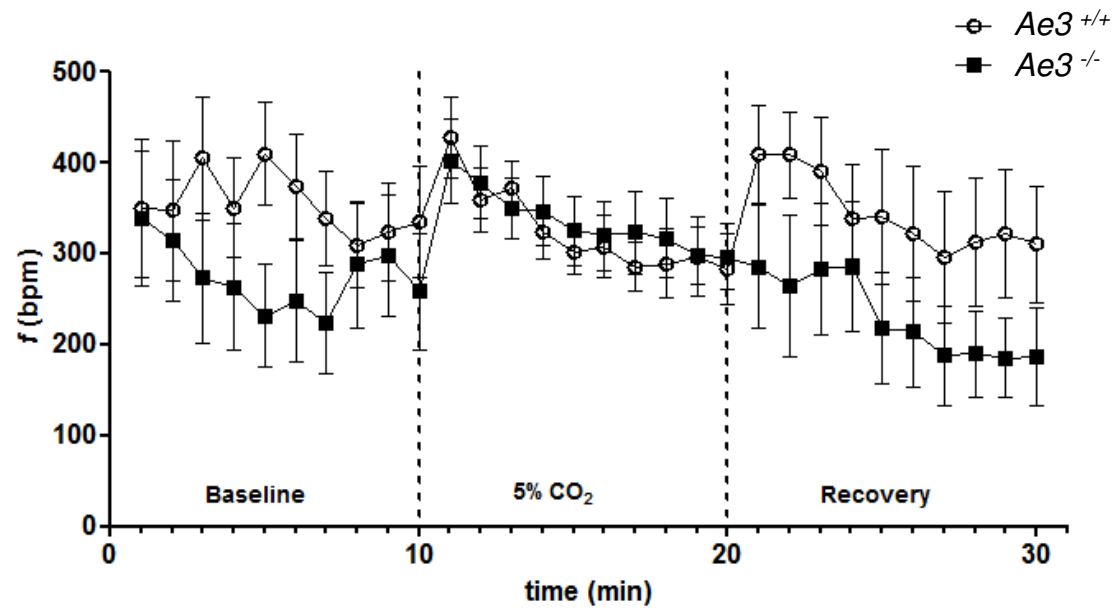


Figure 2

A



B

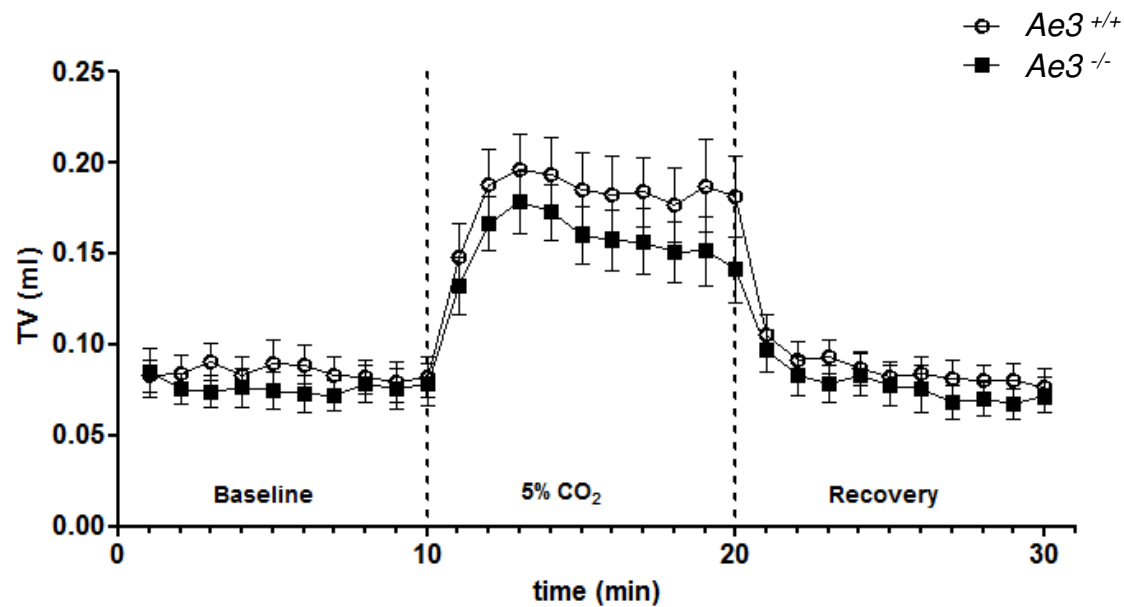
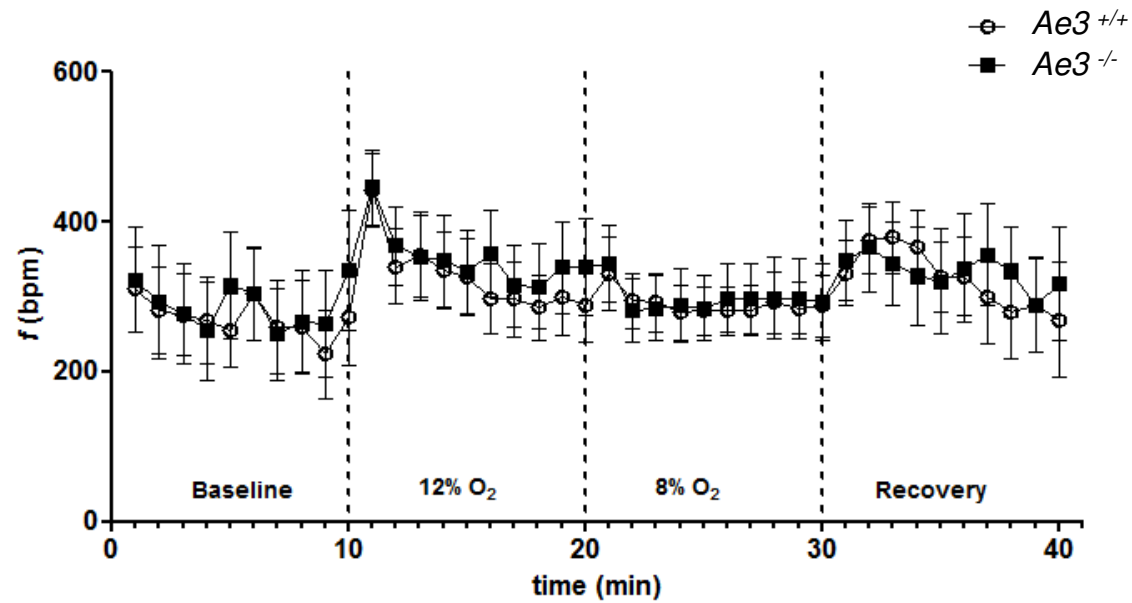


Figure 2

C



D

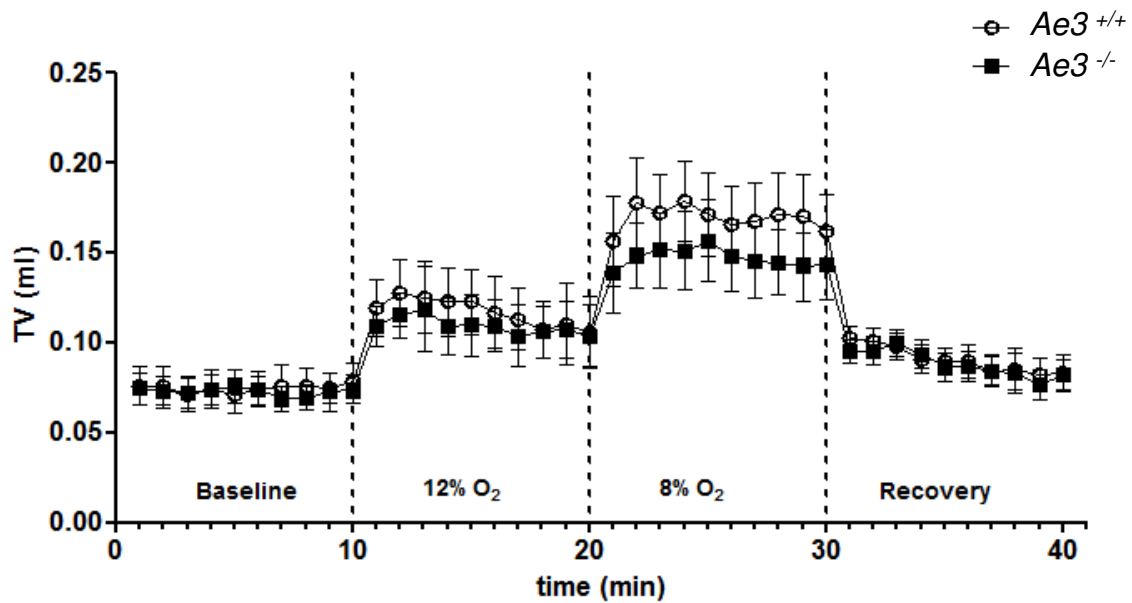
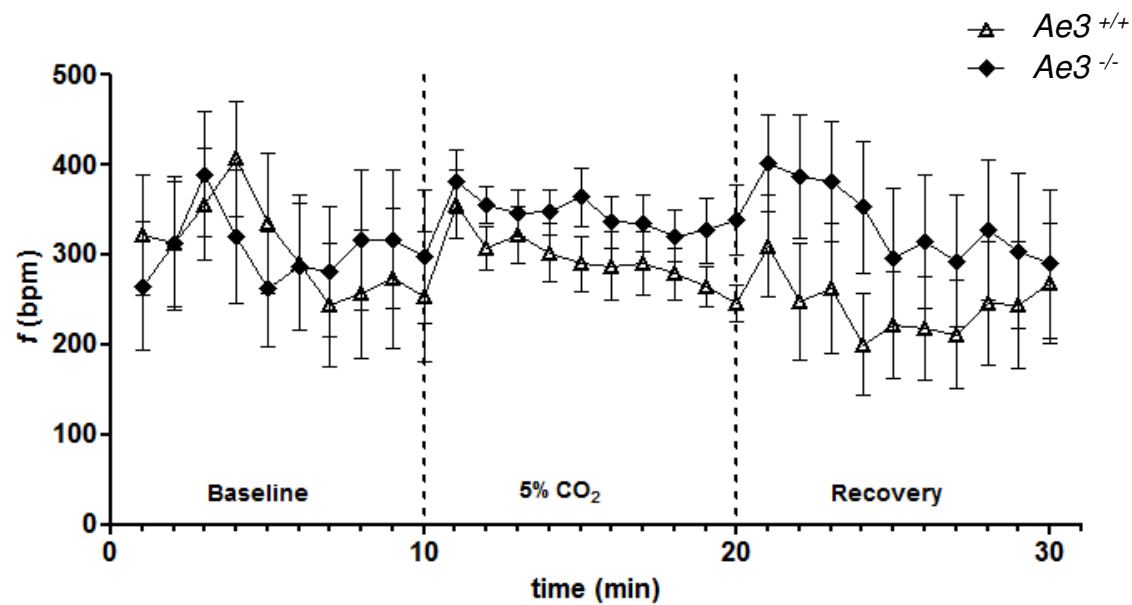


Figure 2

E



F

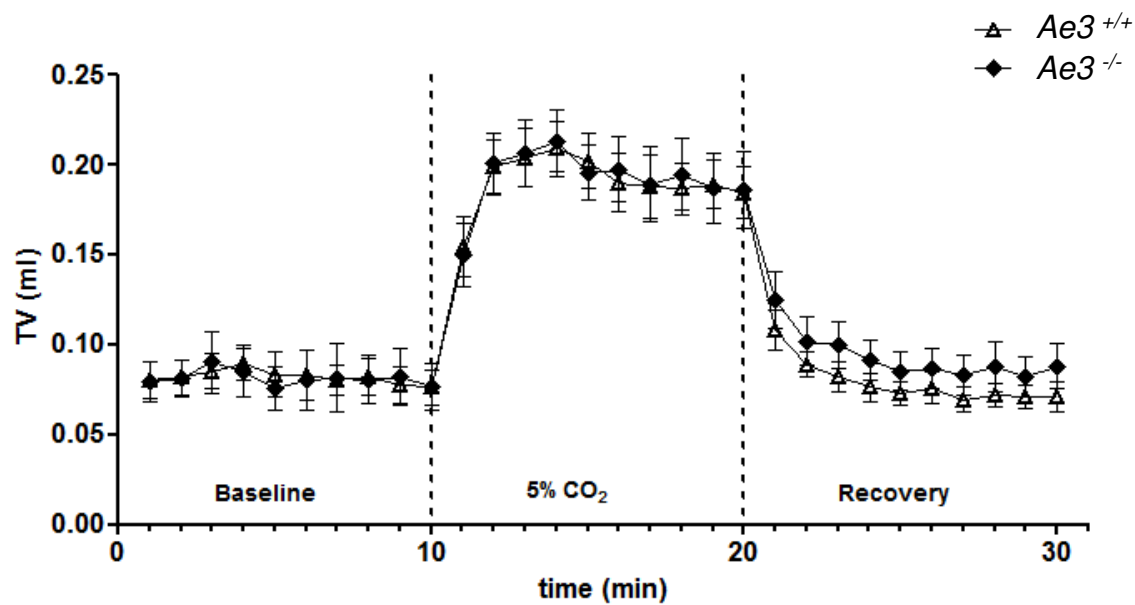
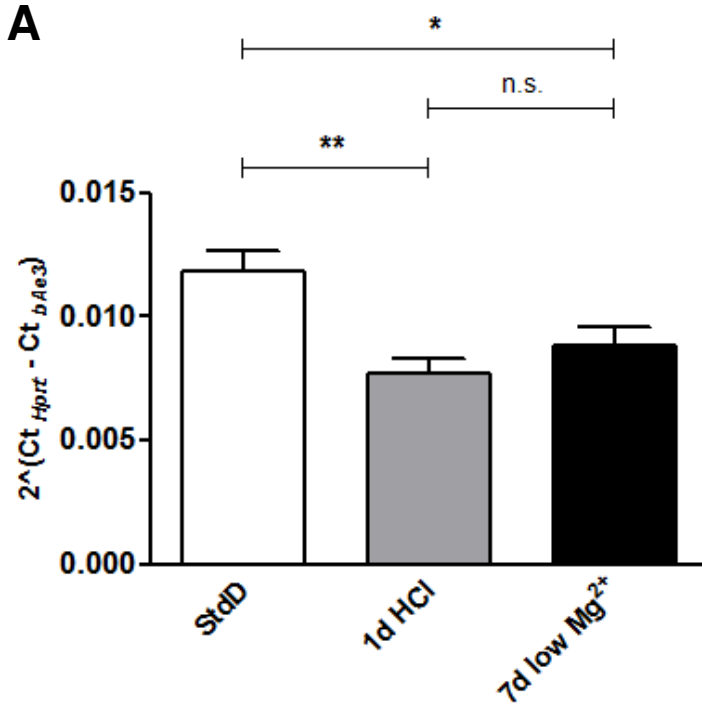
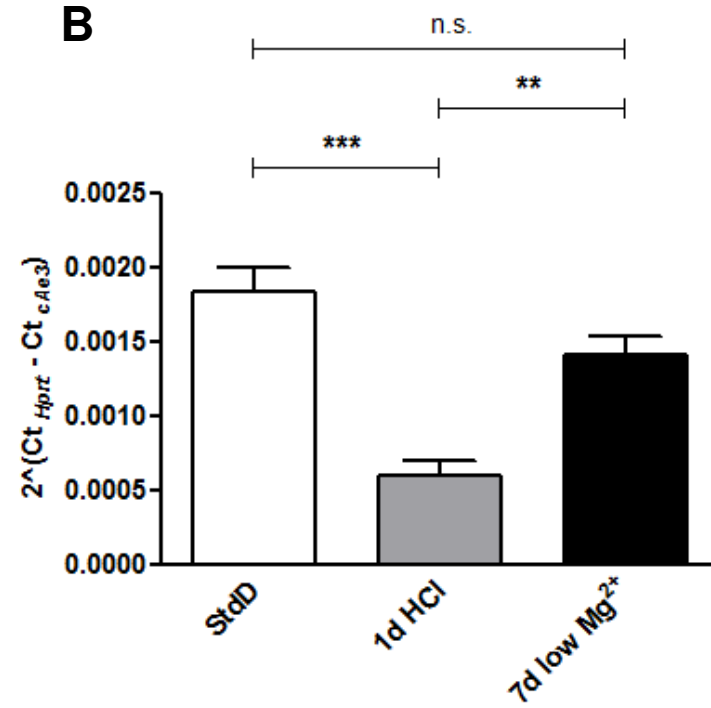


Figure 3

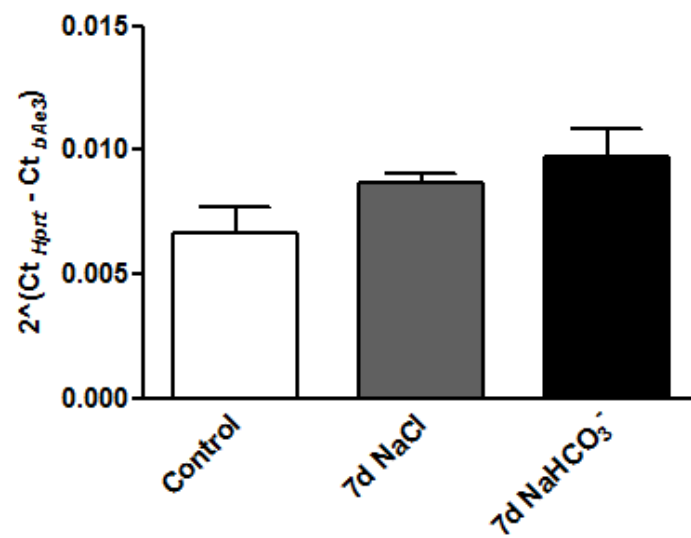
A



B



C



D

